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Search for a Prion-Specific Nucleic Acid

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Diversity of prion strains was attributed to an elusive nucleic acid, yet a search spanning nearly two decades has failed to identify a prion-specific polynucleotide. In our search for a prion-specific nucleic acid, we analyzed nucleic acids in purified fractions from the brains of Syrian hamsters infected with Sc237 prions. Purification of Sc237 prions removed nucleic acids larger than 50 nucleotides as measured by return refocusing electrophoresis (RRGE). To determine the size of the largest polynucleotide present in purified fractions at an abundance of one molecule per infectious (ID_{50}) unit, we measured prions present after inoculation. In order to account for the rapid clearance of prions after intracerebral inoculation, we determined the number of PrP^{Sc} molecules and ID_{50} units of prions that were retained in brain. Factoring in clearance after inoculation, we estimate that the largest polynucleotide present in our purified fractions at one molecule per ID_{50} unit is ≈25 nucleotides in length. In the same fractions, there were ≈3,000 protease-resistant PrP^{Sc} molecules per ID_{50} unit after accounting for clearance of PrP^{Sc} following inoculation. We compared the resistance of Sc237 and 139H prions to inactivation by UV irradiation at 254 nm. Irradiation of homogenates and microsomes diminished prion infectivity by a factor of ≈1,000 but did not alter the strain-specified properties of the Sc237 and 139H prions. The data reported here combined with the production of synthetic prions argue that the 25-mer polynucleotides found in purified prion preparations are likely to be host encoded and of variable sequence; additionally, these 25-mers are unlikely to be prion specific.

Prions cause age-dependent, fatal neurodegenerative diseases, including Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy (25, 49, 78). For many years, the transmissible agents causing these diseases were thought to be slow-acting viruses. Because these infectious agents resembled viruses in many respects, it was difficult to embrace the prion concept when it was initially introduced (55).

That an infectious pathogen might be composed only of a protein seemed most unlikely, particularly when strains of prions were considered (11). As studies of prions began to reveal an unprecedented biology, accommodating the phenomenon of prion strains became increasingly taxing. In the early 1990s, one of us (S.B.P.) proposed that strain-specified characteristics are enciphered in the conformation of PrP^{Sc} (54); subsequently, this idea gained support from experimental studies (10, 37, 42, 50, 65, 74). Yet the notion that a single protein can adopt many different, biologically active conformations remained troublesome. Moreover, only 30 years earlier had the battle to determine the chemical basis of heredity been joined and genes were found to be composed of DNA or RNA but not protein (28, 43).

In mammals, the prion protein (PrP) is encoded by a chromosomal gene and expressed in many tissues, including the central nervous system. By a poorly defined process, the cellular PrP isoform (PrP^{C}) undergoes a profound conformational change to form PrP^{Sc}, the only known component of the infectious prion particle. Limited proteolytic digestion of PrP^{Sc} results in N-terminal truncation of the protein to form PrP 27–30, which retains prion infectivity. A substantial and variable fraction of PrP^{Sc} is protease sensitive and designated $sPrP^{Sc}$; protease-resistant PrP^{Sc} is designated $rPrP^{Sc}$ (65, 66, 76).

To determine if a small polynucleotide was hidden among PrP^{Sc} molecules, we began a search for a prion-specific nucleic acid. In our initial study, we failed to identify a prion-specific polynucleotide in our most purified preparations (46). Still concerned that we had overlooked a nucleic acid molecule, we applied procedures that destroy polynucleotides to fractions highly enriched for prion infectivity as measured by bioassays in rodents (31). Using the technique of return refocusing electrophoresis (RRGE), we were able to fractionate polynucleotide fragments by size and assess their abundance independent of sequence.

As the biology of prions has emerged, the likelihood of an essential nucleic acid molecule seems to grow increasingly remote (57), although a few investigators remain unconvinced (16). Because the absence of a particular molecule in a biologically active preparation is difficult to prove, we undertook yet another set of studies to search for a prion-specific polynucleotide. Moreover, a recent study described an RNA fraction that stimulates the formation of protease-resistant PrP in brain extracts (19). We report here that no prion-specific polynucle-

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otide could be identified in fractions highly enriched for prion infectivity. By measuring the number of infectious (ID_{50}) units and PrP^{Sc} molecules retained in the brain after intracerebral inoculation, we reassessed the particle to infectivity (P/I) ratio and the maximum possible size of a prion-specific polynucleotide. We found that polynucleotides larger than 25 bases were not in sufficient abundance to be present at a P/I ratio of 1 and thus could not be essential for prion infectivity. We also irradiated two prion strains with UV light at 254 nm to determine if one strain was more susceptible to inactivation by UV radiation than the other. Our bioassay results indicate that the two strains were equally resistant to inactivation by UV radiation that was designed to target polynucleotides. Combined with recent studies on the production of synthetic prions that infect either mammals (29, 36, 37, 76) or fungi (34, 38, 70, 73), the results reported here argue persuasively that nucleic acids are not essential either for prion infectivity or for specifying strain-encrypted properties.

MATERIALS AND METHODS

Prion strains and bioassays. The Sc237 strain was obtained from Richard Marsh (40) and was passaged repeatedly in golden Syrian hamsters (Charles River Laboratories, Wilmington, MA). This strain appears to be indistinguishable from strain 263K (32). The 139H isolate, provided by Richard Kimberlin and Richard Carp, was obtained by six passages of mouse 139A prions through golden Syrian hamsters (33). The 139A strain was originally isolated after 20 passages of the Chandler strain in mice (20).

We intracerebrally inoculated groups of eight Syrian hamsters (LVG:Lak; Animal Production Area, Frederick Cancer Research Center, Frederick, MD) with either 139H or Sc237 prions. The animals were euthanized in the terminal stage of disease, and their brains were immediately frozen and stored at −70°C. Brains were homogenized on ice by three 30-s strokes of a PowerGen homogenizer (Fisher Scientific, Pittsburgh, PA) in phosphate-buffered saline (PBS), pH 7.4, containing protease inhibitors (5 mM phenylmethylsulfonyl fluoride and aprotinin and leupeptin at 4 μg/ml each). The resulting 10% (wt/vol) homogenates were spun for 5 min at 500 × *g* in a tabletop centrifuge and the supernatant (S1) was divided in two fractions: one was used for bioassay and the second was mixed 1:1 with 4% Sarkosyl in PBS, pH 7.4, and tested by the conformation-dependent immunoassay (CDI).

For bioassay, three aliquots of 10% brain homogenate were diluted to a final 1% (wt/vol) concentration; 50 μl of this 1% brain homogenate was inoculated intracerebrally into three groups of eight weanling LVG:Lak Syrian hamsters. The titer of Sc237 prions was determined by the incubation-time assay as described (59, 61). We found the same correlation between inoculated dose and incubation time from homogenates, purified prion rods from sucrose gradient centrifugation, and purified prion rods from detergent-lipid-protein complexes (DLPCs), indicating that a relationship between dose and incubation exists (12; unpublished data).

Chemicals and enzymes. Chemicals and solvents from commercial sources were of reagent grade. Lipids were from Avanti Polar Lipids (Alabaster, AL). Benzonase and proteinase K were obtained from Merck (Darmstadt, Germany).

PrP 27–30 and PrP^{Sc} standard purification protocol. PrP 27–30 was purified according to a standard protocol (58). Briefly, a 10% brain homogenate in 0.32 M sucrose was prepared, and the S1 was incubated with Triton X-100 and sodium deoxycholate after low-speed centrifugation. After polyethylene glycol 8000 precipitation, the pellet (P2) was treated with micrococcal nuclease and proteinase K. Ammonium sulfate precipitation and centrifugation resulted in pellet P3, which is the starting material for further nucleic acid separation, either by discontinuous sucrose gradient centrifugation or by ultrafiltration.

In general, the initial purification scheme for PrP^{Sc} resembled that for PrP 27–30 except that proteinase K digestion was omitted and pellet P3 was repeatedly sonicated. Therefore, P3 was adjusted to 20 mg/ml and 200 mM NaCl and sonicated with a microtip eight times for 7 s each at a setting at 6 on a Bronson sonicator for resuspension of PrP^{Sc}. After sonication, the resulting solution was adjusted to 5% Sarkosyl and incubated for 5 min. PrP^{Sc} was centrifuged at 300,000 × *g* (Beckman rotor Ti-50.2) for 30 min. The ultrasonication procedure was repeated three times, so that pellet P6 was the final product.

Modifications to the standard purification protocol. (i) Nucleic acid degradation by benzonase incubation. In order to eliminate nucleic acids at an early step in the purification protocol, three prion preparations (≈160 brains each) were carried out with a benzonase incubation. Benzonase is a recombinant nuclease that degrades all types of nucleic acids down to oligonucleotides of fewer than 20 nucleotides. Brains were homogenized in 0.32 M sucrose according to the standard protocol. Then the homogenate was adjusted to 20 mM Tris acetate, pH 8.3, and 2 mM $MgCl_2$ and subjected to a low-speed spin (JA 10, 4,000 rpm for 30 min at room temperature). Benzonase was added at a concentration of 120 U/ml to the S1, and the mixture was incubated at 37°C for 20 h. All subsequent purification steps were carried out according to the standard protocol.

(ii) Zn^{2+}-catalyzed hydrolysis. Different Zn^{2+} concentrations (4 to 40 mM) and incubation times (1 h, 24 h, and 72 h), in the presence or absence of detergent (β-octyl-glycoside), were tested for their effect on nucleic acid degradation in prion samples. Protein concentrations of 100 μg/ml in 100 mM Tris-HCl, pH 7.0, were incubated at 65°C at the selected Zn^{2+} concentration and for the specified duration. As a control, some samples were incubated at room temperature. After Zn^{2+} hydrolysis, benzonase digestion was carried out at 37°C for 20 h. The samples were boiled in 2% sodium dodecyl sulfate (SDS), digested with proteinase K, organically extracted and precipitated with ethanol, and then analyzed by polyacrylamide gel electrophoresis (PAGE) to observe the effects of different Zn^{2+} concentrations on residual nucleic acids. Aliquots of the samples were inoculated into Syrian hamsters to determine infectivity.

In general, an ethanol precipitation was completed after Zn^{2+} hydrolysis to adjust the sample for the subsequent incubation step, in particular for DLPC formation. In later experiments, cyclen (1,4,7,10-tetraazacyclododecane), a specific chelator for Zn^{2+} ($K_b = 10^{19}\ M^{-1}$), was added at twice the concentration of Zn^{2+} prior to the subsequent ultracentrifugation or DLPC step.

Sucrose gradient centrifugation. The standard procedure for sucrose gradient centrifugation was described previously (31, 46). In contrast to earlier studies, the prion samples from the sucrose gradient fractions were not precipitated by ethanol but were incubated directly, i.e., in the presence of 25% sucrose, with DNase I and 2 mM $ZnCl_2$ at 65°C. The prion sample was "solubilized" by formation of DLPCs and again incubated with nucleases (Bal3l, micrococcal nuclease, and RNase A).

Ultrafiltration. After the standard purification protocol, ultrafiltration can be performed as an alternative to sucrose gradient centrifugation to further purify the sample (27). Pellet P3 was adjusted to 2% Triton X-100 and 0.8% SDS and incubated for 15 min. Ultrafiltration was performed in a tangential filtration device (Ultrasette, Filtron, Northborough, MA) in a continuous mode, a method in which the volume lot is replaced by the addition of fresh buffer throughout the course of filtration. The membrane consists of a modified polyethylenesulfone (PES, omega series), which gives low nonspecific protein binding; it has a molecular size cutoff of 300 kDa. The buffer used was 20 mM Tris-acetate, pH 8.3, and 2% Sarkosyl. The wash volume was approximately 40 to 50 times the starting volume of pellet P3. The final volume for the retentate was between 50 and 100 ml.

Ultracentrifugation. Preparative ultracentrifugation was carried out in a Beckman Optima L80 ultracentrifuge (Beckman Coulter Inc., Fullerton, CA). To reduce the detergent content after ultrafiltration, the prion sample was washed and centrifuged twice. Typically, 1 to 2 ml retentate (≈1 mg protein) was diluted with H_2O to 35 ml in polyallomer centrifuge tubes (Beckman, ≈326823). The centrifugations were performed at 24,000 rpm (≈100,000 × *g*) for 15 h and then 6 h using the SW28 rotor. After completing $CHCl_3$-methanol precipitation, the last step in further nucleic acid degradation, the prion sample was centrifuged in an SW41 rotor at 17,000 rpm (≈55,000 × *g*) for 1 h at 4°C to guarantee quantitative sedimentation.

Nucleic acid analysis by return refocusing gel electrophoresis and silver staining. RRGE was developed to analyze heterogeneous nucleic acids in highly purified prion samples (31, 46). For analysis of the residual nucleic acids in prion preparations, the samples were extracted in chloroform-methanol, followed by boiling in 2% SDS and incubation with 500 μg/ml proteinase K for 3 h. After phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, the samples were loaded onto the RRGE gel.

Gel electrophoresis was carried out in an SE600 gel chamber (Hoefer Scientific, San Francisco, CA). The buffer chamber was filled with 5 liters of running buffer. The glass plates were completely submerged in running buffer at 70°C in order to maintain denaturing conditions. A peristaltic pump recirculated the running buffer from the lower chamber into the upper one.

In this study, RRGE was applied using either a 9% or a 15% polyacrylamide matrix, covering the size range from 13 to 1,100 and 13 to 770 nucleotides, respectively. For each matrix, the gel was cut into seven segments (a to h), and in the case of 9% RRGE, segments b to h were repolymerized at the bottom of a new gel matrix. The cutting edges were 4, 14, 24, 40, 56, 72, and 108 mm for 9%

RRGE and 4, 22, 40, 58, 81, 104, and 127 mm for 15% RRGE, measured from the top. The refocusing runs (250 V) were carried out in the opposite direction and in the presence of 0.5% SDS in order to avoid refocusing of residual proteins. The times for refocusing of different gel segments were between 48 and 42 min for 9% RRGE and 55 min for 15% RRGE. The amount of unknown nucleic acid in the prion sample was determined by comparison with the applied standard.

Nucleic acid polyacrylamide gels were stained with silver according to the method of Sammons (67) and specifically adapted to the requirements of RRGE (0.5% SDS in gel matrix). The gels were washed twice in 10% ethanol and 0.5% acetic acid for 20 min, followed by an incubation in 0.1% $AgNO_3$ for 20 min. They were then washed twice with H_2O for 20 s and developed in 0.008% $NaBH_4$, 1.5% NaOH, and 0.15% formaldehyde. The color reaction was stopped with either 0.75% Na_2CO_3 or 5% acetic acid.

Precipitation of prions by Na/Mg phosphotungstate. The precipitation of both protease-resistant and -sensitive PrP^{Sc} by a polyoxomethalate complex of Na/Mg phosphotungstate (PTA) is described in detail elsewhere (65, 66, 76). Briefly, brains were homogenized by three 15-s strokes to contain final 10% (wt/vol) followed by a clarification spin at 500 × *g* for 5 min. Clarified brain homogenate was then diluted to a final 5% (wt/vol) concentration by PBS, pH 7.4, containing 2% (wt/vol) Sarkosyl, and rehomogenized by three additional 15-s strokes. Samples were mixed with stock solution containing 4% NaPTA and 170 mM $MgCl_2$, pH 7.4, to obtain a final concentration of 0.2 to 0.3% NaPTA (65, 66, 76). Typically, 300-μl samples were incubated for 16 h at 37°C on a rocking platform and centrifuged at 14,000 × *g* in a tabletop centrifuge (Eppendorf, Hamburg, Germany) for 30 min at room temperature. The optional treatment with 25 μg/ml of proteinase K for 1 h at 37°C was performed either before or after precipitation. The pellet was resuspended in H_2O containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and aprotinin and leupeptin at 2 μg/ml each) and assayed by CDI.

Sandwich CDI for proteinase K-resistant PrP^{Sc}. The principle, development, calibration, and calculation of PrP^{Sc} concentration from CDI data were described in detail elsewhere (65, 66, 76). An important modification to the original protocol was the introduction of a sandwich format using the recombinant antibody fragment (Fab) R1 as a capture antibody (51). Briefly, each sample was divided into two aliquots: (i) untreated and designated native and (ii) mixed to a final concentration of 4 M guanidinium HCl, heated for 5 min at 80°C, and designated denatured. Both samples were immediately diluted 20-fold with H_2O containing protease inhibitors (5 mM phenylmethylsulfonyl fluoride and aprotinin and leupeptin, 4 μg/ml each), and aliquots were loaded on a 96-well polystyrene plate that was previously saturated overnight with purified recombinant Fab R1 in PBS, pH 7.4 (51). The plates were incubated for 2 h at room temperature and then blocked with Tris-buffered saline (TBS, pH 7.8) containing 0.5% (wt/vol) bovine serum albumin and 6% (wt/vol) Sorbitol for 2 h at room temperature.

In the next step, they were washed three times with TBS, pH 7.8, containing 0.05% (vol/vol) Tween 20 and incubated at room temperature for 2 h with europium (Eu)-labeled monoclonal immunoglobulin G 3F4. The plates were developed after seven washing steps in enhancement solution provided by the Eu label supplier (Wallac Inc, Turku, Finland), and the signal was counted on a Discovery dual-wavelength, time-resolved fluorometer (Packard Instruments, Meriden, CT). All the data described in this paper were generated with Eu-labeled monoclonal antibody 3F4, described elsewhere (30). We used denatured recombinant β-sheet SHaPrP(90–231) (45) as a standard. The concentration of $rPrP^{Sc}$ was calculated after PTA precipitation and proteinase K treatment from the published formula (65, 76).

UV irradiation. Ten-percent (wt/vol) brain homogenate was prepared in PBS using a disposable homogenizer (Sage Products Inc.). After a short centrifugation (500 × *g* for 10 min), the supernatant was used as a "homogenate sample" or further purified as a microsomal fraction. The clarified homogenate was mixed in a 1:10 ratio with ice-cold water. After 30 min of incubation on ice with stirring, the preparation was centrifuged at 100,000 × *g* for 60 min. The resulting pellet was resuspended in PBS and the hypotonic step was repeated. The final microsomal membranes were resuspended in 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Triton X-100. Samples of 0.5 ml from the homogenate and microsomal membranes were distributed in a 24-well microtiter plate (Falcon) with a surface area of 2 cm^2. The plates were irradiated at 4°C at 10 cm from the UV light source with an E_{max} of 254 nm.

The P3′SS vector was irradiated with 19.5 J/m^2 (15 s) and 39 J/m^2 (30 s), after which it was purified using the QIAGEN genomic purification kit. DNA was incubated with T4 den V to cleave UV photoproducts. The percentage of undamaged plasmid was calculated from the ratio of form I (uncut) to total plasmid in 1% agarose gels. Quantification was completed by densitometry of the gel negatives.

Calculation of nucleic acid particle per infectious unit. The P/I ratio was calculated based on the following formula: $P/I = Q \times N_0/330 \times I \times S \times L$, where Q is the experimentally determined amount of residual nucleic acid in the prion sample (in g), N_0 is Avogadro's number (6.02×10^{23}), 330 is the molecular weight of one nucleotide, I is the total infectivity from the corresponding experiment, S is the number of different species per gel segment, and L is the average length in nucleotides. An even distribution of all nucleic acid species over the range of analysis is assumed.

RESULTS

Our earlier analyses of residual nucleic acids in purified fractions of scrapie prions were performed on samples prepared according to the "standard protocol" (31, 46), which consists of brain homogenization followed by detergent extraction, enzymatic digestions, differential centrifugation, and sucrose gradient centrifugation. To remove the prions from the sucrose, ethanol precipitation was routinely used. To minimize residual nucleic acids, enzymatic digestion and Zn^{2+} hydrolysis were used followed by DLPC formation with consecutive nuclease digestions. After deproteinization of the samples, the remaining nucleic acids were analyzed by RRGE.

To increase the recovery of prion infectivity and reduce the amount of residual nucleic acid, we investigated the order of nuclease addition, conditions for Zn^{2+} hydrolysis, and the use of ultracentrifugation in place of ethanol precipitation. In another series of experiments, we substituted ultrafiltration for sucrose gradient centrifugation.

Nucleic acid degradation prior to prion rod formation. Because nucleic acids might be trapped during prion rod formation and thus protected against nucleic acid degradation, we attempted to degrade nucleic acids before rod formation. Prion rods form when PrP^{Sc} is converted to PrP 27–30 by limited proteolysis in the presence of detergent (44). Although the recovery of prion infectivity in these experiments was good, the protein concentration was so high that nucleic acid analysis was problematic. Digestion of genomic DNA with benzonase apparently releases chromosomal proteins that were otherwise separated from the prions by a low-speed centrifugation that sediments much of the nuclear DNA. These problems prevented us from pursuing this approach after three experiments.

Ethanol precipitations. The "standard protocol" reported earlier included two ethanol precipitations: one after sucrose gradient fractionation and another after Zn^{2+} hydrolysis. These precipitations were used to change the buffer and volume for subsequent processing. Because bioassays repeatedly indicated a decrease in prion infectivity after ethanol precipitation, ultracentrifugation was used to sediment the prions. In Table 1, the recoveries of prion infectivity after ethanol precipitation are compared to those after ultracentrifugation at ≈100,000 × *g* for 6 or 15 h. The recovery of prion infectivity after ultracentrifugation was generally 10-fold better than by ethanol precipitation.

Zn^{2+}-mediated hydrolysis of polynucleotides. Zn^{2+}-catalyzed hydrolysis of RNA is a well-documented reaction. In our earlier studies, we employed 2 mM Zn^{2+} but in the studies described here, we varied the Zn^{2+} concentration from 4 to 40 mM and incubated the samples from 24 to 72 h. Maximal degradation of RNA occurred after 24 h of incubation at 65°C with 20 mM Zn^{2+}. Under these conditions, prion infectivity

TABLE 1. Recovery of prion infectivity in samples subjected to ethanol precipitation or ultracentrifugation[a]

Prion fraction/purification step	Total titer (log ID_{50}) before concentration ± SEM	Total titer (log ID_{50}) after concentration ± SEM	Total titer (log ID_{50}) after DLPC/nucleases ± SEM
Sucrose gradient fraction/ethanol precipitation[b]	10.1 ± 0.5	8.2 ± 0.3	8.3 ± 0.4
Sucrose gradient fraction/ultracentrifuged[c]	9.6 ± 0.2	9.1 ± 0.3	8.5 ± 0.2
Sucrose gradient fraction/ultracentrifugation (modified protocol)	9.9 ± 0.4	8.5 ± 1.0	9.0 ± 0.5
Retentate after ultrafiltration	9.9[d]	7.8 ± 0.8	8.7 ± 0.6

[a] Values represent averaged values from eight or three independent experiments.
[b] Data from Meyer et al. (46) for eight experiments.
[c] Data from Kellings et al. (31) for three experiments.
[d] The three samples tested originated from the same prion fraction.

remained stable (data not shown). When we performed benzonase incubation subsequent to Zn^{2+} hydrolysis, the titer also remained unchanged.

Ultrafiltration. We investigated whether ultrafiltration might be substituted for sucrose gradient centrifugation. The retentate fraction after ultrafiltration did not contain sucrose but did possess >99.9% of the initial prion infectivity. Since Sarkosyl in the retentate is incompatible with subsequent nucleic acid degradation by Zn^{2+} hydrolysis, a second ultrafiltration was performed without Sarkosyl. Unfortunately, this second ultrafiltration step reduced the prion infectivity by a factor of ≈100. This loss in infectivity was unacceptable, and so the protocol using a second ultrafiltration to remove Sarkosyl was abandoned.

Based on the foregoing findings, the P3 fraction was resuspended in a buffer containing 2% Sarkosyl and subjected to ultrafiltration. After reducing the volume of the retentate from 500 to 50 ml, aliquots of 1 to 2 ml were diluted to 35 ml in H_2O. The samples were then sedimented at 100,000 × *g* for 15 h, resuspended in 35 ml H_2O and sedimented again at 100,000 × *g* for 6 h. After these washes, prion infectivity was quantitatively recovered in the sample in the absence of Sarkosyl (Table 1).

Retentate fraction analysis for nucleic acid. For nucleic acid degradation, 20 mM Zn^{2+} was used as described above followed by benzonase digestion. To remove the free Zn^{2+} ions from solution prior to benzonase digestion, the Zn^{2+} was chelated with cyclen. We analyzed three samples KK-D5, KK-D6, and KK-D7 by 9% RRGE. An example of RRGE analysis is shown in Fig. 1.

Because PrP^{Sc} forms amorphous aggregates and does not assemble into prion rods, we analyzed purified prion fractions containing full-length PrP^{Sc} (sample KK-D7) for nucleic acids. Dispersion of the prions into DLPCs was omitted. RRGE analysis of this preparation containing full-length PrP^{Sc} revealed that the amount of residual nucleic acid was ≈20-fold higher than that in preparations containing PrP 27–30. The prion infectivity of this preparation containing full-length PrP^{Sc} was $10^{7.8}$ ID_{50} units/ml, which was slightly lower than the titer of preparations containing PrP 27–30. The maximum length for a hypothetical prion-specific nucleic acid in the PrP^{Sc} preparation yielded a value of 200 nucleotides. This large number of nucleotides caused us to abandon the use of preparations containing full-length PrP^{Sc}.

The conditions employed for sample KK-D6 gave the most effective nucleic acid degradation and thus were repeated two more times to produce samples KK-D8 and KK-D9. The corresponding total prion infectivity levels in the three samples were $10^{8.0}$, $10^{8.8}$ and $10^{9.2}$ ID_{50} units, respectively. These samples contained ≈100-fold more prion infectivity than previously obtained by ultracentrifugation using the "standard protocol." From the RRGE analysis, the amounts of nucleic acids within the different size ranges were estimated by comparing the intensities of the bands to those of the marker DNAs (Fig. 1). The best quantification was obtained using the original gels; photographic reproduction, as shown in Fig. 1, was inferior. If a band was not visible, the lowest concentration of a visible marker DNA was taken.

The total amount of residual nucleic acids was in the range of 10 to 20 ng. Taking the titers into account, the P/I ratio for a hypothetical prion-specific nucleic acid could be calculated depending upon the assumed size. Plots are shown in Fig. 2A for the KK-D6, KK-D8, and KK-D9 samples. The line for a P/I ratio of 1 represents the most extreme assumption: one molecule is sufficient for infection. Nucleic acids below the line cannot be essential for infectivity, whereas those above the line cannot be excluded. Although the specific infectivity of retentates after ultrafiltration was approximately 100-fold lower than those from sucrose gradient fractions, the P/I ratios for these experiments were similar. The maximum lengths of pu-

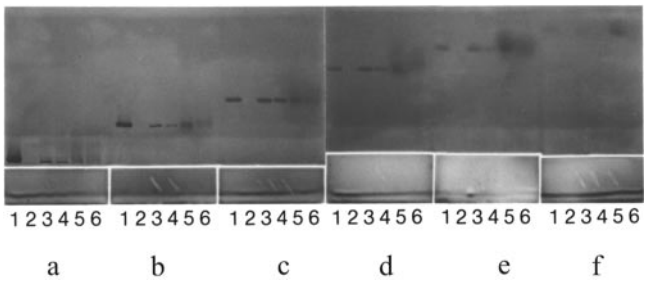

FIG. 1. Analysis of residual nucleic acids in preparations of PrP 27–30 by 15% RRGE. The modified protocol was applied to sucrose gradient fractions. Nucleic acids of the following size ranges are refocused: (a) 767 to 103 nucleotides; (b) 102 to 54 nucleotides; (c) 53 to 33 nucleotides; (d) 32 to 21 nucleotides; (e) 20 to 13 nucleotides; and (f) smaller than 13 nucleotides. Lanes: (1) 200 ng sonicated calf thymus DNA; (2) benzonase as a control; (3) marker DNA (5 ng 400 nucleotides in a, 5 ng 100 nucleotides in b, 5 ng 39 nucleotides in c, 10 ng 28 nucleotides in d, 10 ng 17 nucleotides in e, 20 ng 12 nucleotides in f); (4) marker DNA as in lane 3 but (2 ng in a, 2 ng in b, 2 ng in c, 5 ng in d, 5 ng in e, 5 ng in f); (5) prion sample KK-G5; (6) prion sample KK-G5 plus benzonase incubation prior to RRGE analysis, showing a more than 90% signal reduction.

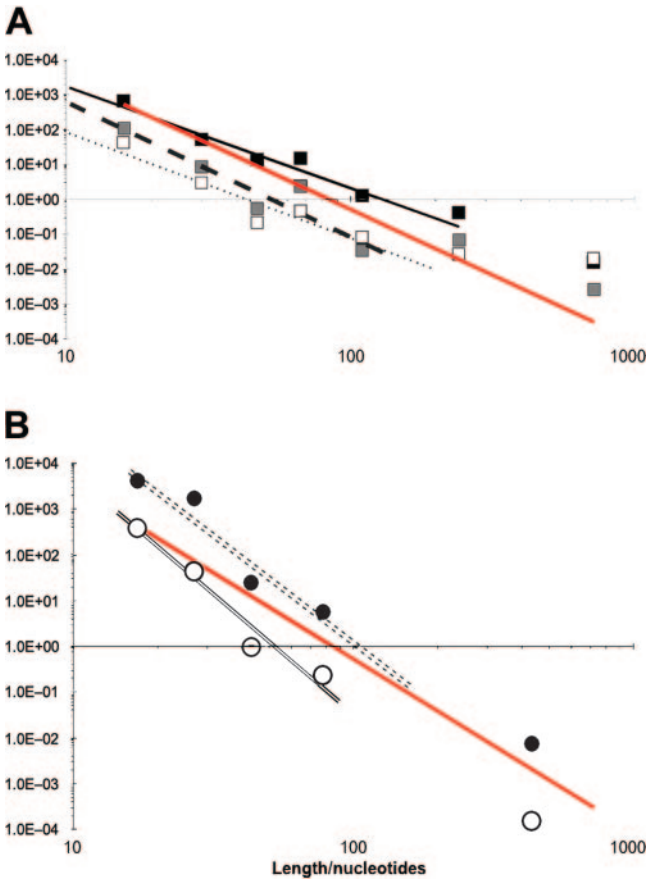

FIG. 2. Particle-to-infectivity ratio plotted as a function of the average length of the residual nucleic acid. (A) Three independent preparations, KK-D6 (black squares); KK-D8 (gray squares), and KK-D9 (white squares), were analyzed, which were prepared according to the modified protocol and applied to the retentate after ultrafiltration. Linear regression (LR) for the empirical relation between log P/I and log length/nucleotides: D6-LR, solid line; D8-LR, dashed line; D9-LR, dotted line. (B) Two independent preparations, KK-G5 (black circles) and KK-G6 (white circles), were analyzed, which were prepared according to the modified protocol and applied to sucrose gradient fractions. G5-LR, double-dashed line; G6-LR, double-solid line. The published data (red lines) are from reference 31.

tative prion-specific nucleic acids were 45, 54, and 129 nucleotides.

The above calculations assume that all the prions and accompanying nucleic acids remain in the brain of the bioassay animals and initiate infection. Assuming the P/I ratio is not altered upon inoculation, the clearance of prions from the brain will affect the maximum length of the potential prion-specific nucleic acid. As described below, the size of such putative nucleic acids must be adjusted to reflect the clearance from the brain.

Sucrose gradient fraction analysis for nucleic acid. Encouraged by the foregoing results using the retentate obtained by ultrafiltration, we repeated the nucleic acid degradation procedures using sucrose gradient fractions with high specific infectivity. Since sucrose gradient fractions contain ≈50% sucrose, aliquots were diluted twofold. Both Zn^{2+}-mediated hydrolysis and benzonase digestion were carried out in the presence of 25% sucrose (samples KK-G1 to -G4). Under these conditions, we found that Zn^{2+} seems to precipitate nucleic acids and thus protects them from further degradation. Based on this finding, we omitted the Zn^{2+} hydrolysis from subsequent studies. RRGE analysis of the sample labeled KK-G5 is shown in Fig. 1 and the P/I ratios for samples KK-G5 and -G6 experiments are depicted in Fig. 2B. The maximum length of a putative prion-specific nucleic acid in six samples (KK-G1 to KK-G6) was between 53 and 116 nucleotides.

We next compared the prion infectivity in purified fractions derived from sucrose gradient ultracentrifugation and from ultrafiltration. Each step used to reduce nucleic acid in these purified fractions is listed in Table 2 along with the prion titers as measured by bioassay in rodents. Prions in fractions purified by sucrose gradient centrifugation behaved quite differently from those purified by ultrafiltration with respect to infectivity after exposure to Sarkosyl or ethanol. While the total infectivity of the retentate fraction could be reactivated after DLPC formation, the titers of the sucrose gradient fraction continuously declined. This finding contrasts with earlier data in which infectivity in sucrose gradient fractions purified by the "standard protocol" increased ≈10-fold upon dispersion into DLPCs (22).

Clearance of prions upon inoculation into brain. The P/I ratio for PrP^{Sc} molecules per ID_{50} unit was originally estimated

TABLE 2. Changes in infectivity during purification for sucrose gradient and retentate fractions[a]

Sucrose gradient material			Retentate after ultrafiltration		
Purification step	Avg log ID_{50} (total)	Avg log ID_{50} units/ml	Purification step	Avg log ID_{50} (total)	Avg log ID_{50} units/ml
Sucrose gradient fraction (10 μg/ml)	11.1	9.5	Pellet P5 (retentate) (1 mg/ml)	8.1	8.3
25% sucrose	10.2	8.3	n.a.	n.a.	n.a.
Zn^{2+} hydrolysis	9.9	7.9	Zn^{2+} hydrolysis	6.8	7.0
Benzonase digestion	9.8	7.8	Benzonase digestion	6.7	6.9
DLPC resuspension	9.2	8.7	n.a.	n.a.	n.a.
DLPC formation (100 μg/ml)	9.0	8.6	DLPC formation (1 mg/ml)	9.2	8.3
DLPC nuclease 1	8.8	8.4	DLPC nuclease 1	8.9	8.1
DLPC nuclease 2	9.0	8.7	DLPC nuclease 2	8.4	7.5
Chloroform ppt	8.7	9.2	Chloroform ppt	7.5	7.9
SDS boiling	8.4	8.8	SDS boiling	7.1	7.5
PK digestion	7.9	8.3	PK digestion	7.1	7.4

[a] Abbreviations: n.a., not applicable; ppt, precipitation; nuclease 1 and 2, two subsequent incubations with nuclease Bal31, micrococcal nuclease, and RNase A; DLPC formation means after 20 min of sonication; PK, proteinase K.

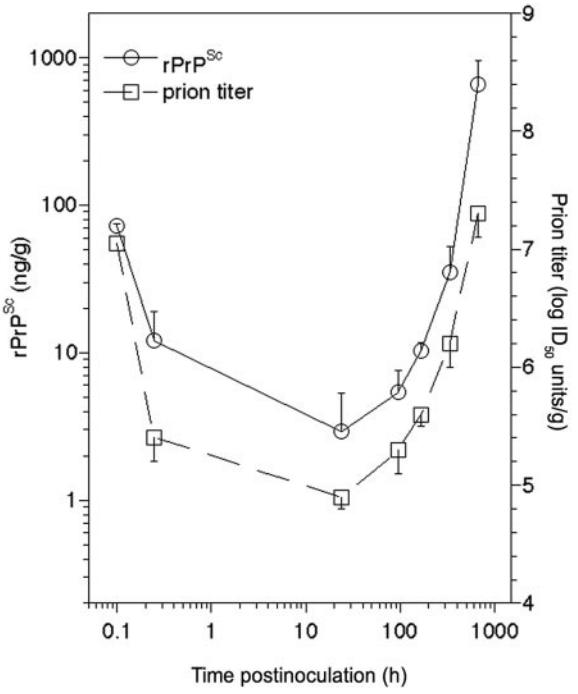

FIG. 3. Parallel clearance of infectivity and levels of $rPrP^{Sc}$ after intracerebral inoculation of Sc237 prions in Syrian hamsters. An infectivity titer of $10^{7.05}$ ID_{50} units was injected. The data points for infectivity are means ± standard error of the mean from three independent incubation-time assays, each performed in eight Syrian hamsters; the $rPrP^{Sc}$ values are means ± standard error of the mean from CDI measurements in eight different Syrian hamster brains, each performed in duplicate.

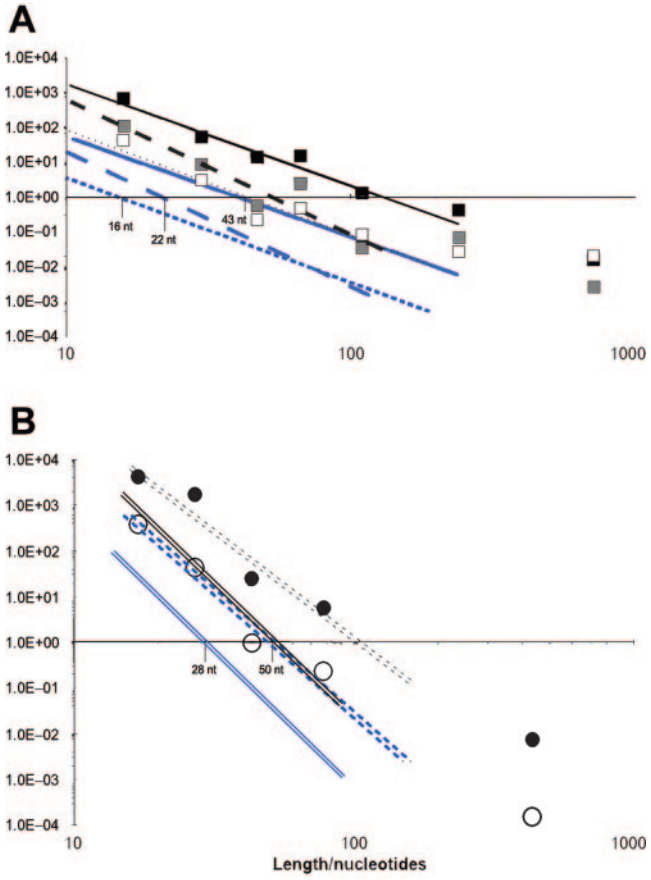

FIG. 4. Effect of clearance on the nucleic acid particle/infectious unit ratio in dependence upon the average length of the residual nucleic acid. According to 96% clearance (cf. Fig. 3), all original data in Fig. 2 are reduced to 4% and redrawn to determine the length of the residual nucleic acid with a P/I ratio of 1. The data from Fig. 2A (A) and from Fig. 2B (B) are corrected for the clearance of prions (blue lines). Symbols are as in Fig. 2. Panel A: D6-CL, blue solid line; D8-CL, blue dashed line; D9-CL, blue dotted line. Panel B: G5-CL, blue double-dashed line; G6-CL, blue double-solid line.

using fractions purified by sucrose gradient centrifugation (60). That analysis indicated that the P/I ratio is $\approx 10^5$. This estimate assumed that all of the PrP 27–30 intracerebrally inoculated into bioassay rodents gave rise to prion infectivity; in other words, all of it was retained in the brain.

In the studies presented here on the kinetics of PrP^{Sc} retention in the brain after intracerebral inoculation of Syrian hamsters, we found that less than 10% of PrP 27–30 in the injected inoculum remained in the brain. This result should not have been surprising since earlier studies on India ink particles and bacteriophage showed similar levels of retention in the brains of mice (13, 69).

Groups of eight Syrian hamsters were inoculated intracerebrally with Sc237 prions in SHa brain homogenate that contained $\approx 10^{7.1}$ ID_{50} units per 50-μl dose. The hamsters were euthanized at various times after inoculation and their brains removed and homogenized in PBS to create a 10% (wt/vol) suspension. The level of prions in homogenate at each time point was measured by bioassay and level of $rPrP^{Sc}$ determined by the CDI.

During the first 24 h after intracerebral inoculation, prion infectivity in the brain declined ≈99% and the level of $rPrP^{Sc}$ decreased ≈96% (Fig. 3). By 96 h, the levels of infectivity and $rPrP^{Sc}$ were increasing; this indicates that accumulation of apparently newly formed $rPrP^{Sc}$ had begun.

Whether the PrP^{Sc} detected at 24 h is a mixture of newly formed and inoculated $rPrP^{Sc}$ molecules is unclear. Assuming that all of the $rPrP^{Sc}$ in the brain after 24 h comes from the inoculum, we estimate that only 4% of the inoculated $rPrP^{Sc}$ remains at this time. The 50-μl inoculum contained 72 ± 3 ng of $rPrP^{Sc}$ while the brains (≈1 g) of hamsters sacrificed 24 h after inoculation contained 2.9 ± 2 ng of $rPrP^{Sc}$, as determined by the CDI.

If we assume that between 96 and 99% of the prions in the inoculum exit from the brain within the first 24 h after inoculation, then it is reasonable to reduce the P/I ratio by a factor between 25 and 100. After recalculation, the P/I ratio for $rPrP^{Sc}$ molecules per ID_{50} unit is between 1,000 and 4,000.

Similarly, the number of potential nucleic acid molecules per infectious unit has to be corrected for the clearance effect. If we assume that the residual nucleic acids in the purified inoculum exit from the brain during the first 24 h at the same rate as $rPrP^{Sc}$, then we must adjust the calculation of the maximum size of a polynucleotide present in our purified fractions at an abundance of one per ID_{50} unit. The interpolating straight lines in Fig. 2 have to be shifted to account for the clearance of 96% of the inoculum (Fig. 4). Based on these plots, we esti-

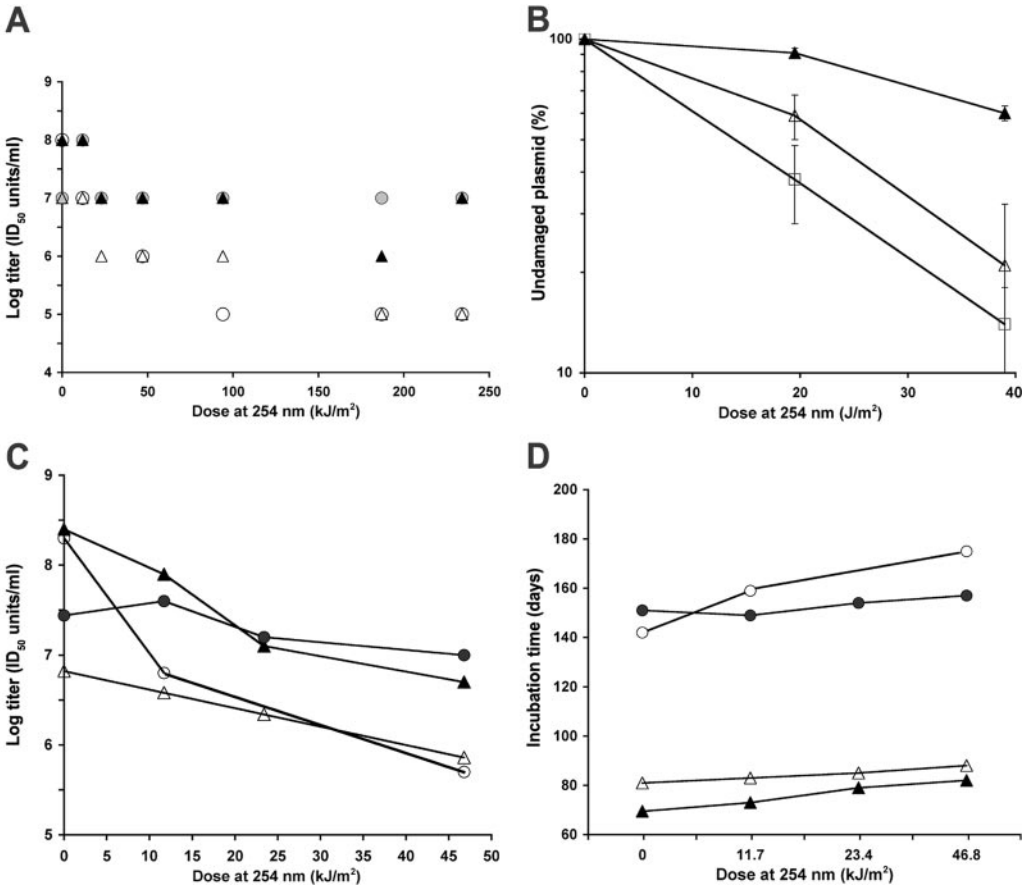

FIG. 5. UV inactivation of two scrapie strains and a plasmid as a control. (A) Summary of infectivity data for strains Sc237 and 139H after irradiation up to 239 kJ/m^2. (B) Dose reduction from shielding of microsomes and homogenates as used in panel A, analyzed by the UV inactivation of a plasmid restriction site. (C) Redrawing of the low close range (0 to 50 kJ/m^2) of panel A. (D) Incubation times up to onset of symptoms in the low-dose range as in panel C. For all panels: open circles, 139H microsomes; solid circles, 139H homogenate; open triangles, Sc237 microsomes; solid triangles, Sc237 homogenate; open squares, buffer. Prion titers in panels A and C were calculated from the incubation times as described in Materials and Methods.

mate that the maximum length of a putative, prion-specific polynucleotide is 25 nucleotides.

UV irradiation at 254 nm. Homogenates and microsomes harboring the Sc237 and 139H strains were irradiated with UV light (254 nm) up to 1,200 kJ/m^2. Prion inactivation was measured by bioassay. The dose-effect curves with a logarithmic scale for the titer were nonlinear, whereas a linear relationship is expected for a simple, one-hit inactivation mechanism (Fig. 5A). After an initial decrease up to about 50 kJ/m^2, the curves nearly level off.

We observed two effects that might be responsible for the nonlinearity. First, the solutions exhibited a fairly high optical density, i.e., the homogenate was even-colored. Due to the high optical density, the samples could not be irradiated homogeneously, which could have been overcome by stirring the sample, but this was not performed for technical reasons. This feature led to particular problems in connection with a second effect, the insolubility of the prion preparations, because the infectious material sedimented during irradiation into regions of higher optical density and consequently lower irradiation intensity. If one takes into account that the high doses were achieved due to long irradiation times, the lower efficacy of the irradiation after 1 h can be explained.

A control experiment was performed measuring damage to the restriction sites in plasmids. The results of this study support the interpretation given above. Plasmid damage was studied in buffer and compared to plasmids added to brain homogenate and microsome fractions, which were prepared like those used in Fig. 5A. The homogenate shielded the plasmids from the UV irradiation by a factor of ≈5, whereas the shielding by microsomes was smaller (Fig. 5B). We were unable to interpret these results quantitatively, and the UV optical density of the homogenate or microsome fraction might vary too much from preparation to preparation for us to be able to correct its influence on the data in Fig. 5A. Control experiments showed that prolonged irradiation of homogenate or microsomes was difficult to evaluate quantitatively.

If the dose-effect curves were evaluated for only up to 1 h of irradiation time, more reliable results were obtained (Fig. 5C). These data are affected by a relatively high error rate of every single point, but the errors did not depart from a linear relationship. Fitting the data results in D_{37} values between 8 and 24

kJ/m^2 for the two strains; moreover, no difference in resistance to inactivation by UV irradiation at 254 nm could be detected between the two strains. The range of D_{37} values is similar to those two of us (J.E.C. and S.B.P.) reported in an earlier study in which the D_{37} values varied from 17 to 22 kJ/m^{-2} (9). In Fig. 5D, the incubation times are depicted as obtained directly from the experiments.

DISCUSSION

The search for a prion-specific nucleic began four decades ago. Transmission of scrapie to mice ushered in the application of biochemical experimentation aimed at revealing the composition of the infectious pathogen (14, 53). The startling results of ionizing and UV irradiation studies performed on mouse brain homogenates intensified the search for the nucleic acid genome of the putative "virus" causing scrapie (3, 4, 35). Those studies argued that the scrapie agent does not contain a nucleic acid, because it is extremely resistant to inactivation by irradiation at 254 nm and its target size, determined by ionizing radiation, is less than 150 kDa.

To search for a prion-specific nucleic acid, several different approaches have been taken. First, numerous procedures that hydrolyze or modify nucleic acids have been used to probe for the existence of a scrapie-specific nucleic acid (21, 53, 55). No procedure that hydrolyzes or modifies nucleic acids but does not alter proteins has been shown to reduce the titer of prions. Second, molecular cloning and differential hybridization studies failed to identify a prion-specific polynucleotide (2, 48). Third, in biophysical studies searching for a small, prion-specific nucleic acid similar to a viroid, these nucleic acids were excluded (31, 46). Additionally, numerous unpublished studies have been performed in search of a prion-specific nucleic acid, but none has been found.

Naturally occurring prions. Unable to find evidence for a scrapie-specific nucleic acid in partially purified fractions prepared from Syrian hamster brains, the prion concept was introduced (55, 56). The possibility of a host-encoded, infectious protein along with several other scenarios was set forth to explain the apparent absence of a nucleic acid genome within an infectious pathogen. Opposition to the prion hypothesis was stout, but the opponents never identified the nucleic acid genome that they so vociferously championed (7, 11, 16, 39, 47, 63).

The resistance of purified prions to inactivation by procedures that hydrolyze or modify nucleic acids suggested that the putative genome of the prion was either quite small or did not exist. This resistance to inactivation raised the possibility that the prion genome was not only small but also well protected. Small, spherical viruses such the polio- and parvoviruses were known to be much more resistant to inactivation than larger viruses such as the one causing herpes (64, 68). But scrapie prions are much more resistant to inactivation by procedures that hydrolyze or modify nucleic acids than the sturdiest virus.

Faced with inactivation studies that argued for the absence of a genome in the prion, two of us (S.B.P. and D.R.) began a series of studies designed to identify a prion-specific nucleic acid using biophysical techniques that could detect relatively small nucleic acids such as viroids (46). Unable to find a prion-specific nucleic acid, we developed a technique to increase the sensitivity of electrophoretic separations that we called RRGE (31). Here, we report that RRGE was unable to identify a prion-specific nucleic acid but was able to exclude a polynucleotide of more than 25 bases (Fig. 4).

Synthetic prions. Although the foregoing studies argue for the absence of a prion-specific polynucleotide, proving a negative is always difficult. The recent production of mammalian and fungal synthetic prions provides positive evidence for the absence of a prion-specific nucleic acid (29, 34, 36–38, 70, 73, 76). Synthetic peptides or purified recombinant proteins acquired infectivity upon being folded into β-sheet–rich conformations. When wild-type, recombinant PrP was polymerized into amyloid fibrils, the protein acquired a high β-sheet content as well as infectivity (6, 36). When the 55-mer mutant peptide was induced into a β-rich conformation, it acquired infectivity (29, 76). The same peptide in a non-β-rich conformation was not infectious.

For a prion-specific nucleic acid to be present in synthetic prion preparations that infect mammals or fungi, it must be an omnipresent contaminant. In the case of mammalian prions, the putative nucleic acid must contaminate the chemicals used to produce synthetic peptides as well as *Escherichia coli* cultures from which recombinant PrP was purified. This scenario seems most improbable, especially in view of the large number of controls performed in studies of both mammalian and fungal synthetic prions.

Strains of prions. Although considerable effort has been expended in the search for a small nucleic acid, the inability to find one always left open the possibility that an elusive polynucleotide is responsible for the strain-specified properties of prions. The existence of strains has been used to argue for a nucleic acid as the informational molecule of the prion (11, 16). The proposal that strain-specified biological information might be carried in the conformation of PrP^{Sc} was met with resistance (54), particularly since proteins were thought to possess only one biologically active conformation (5). Subsequently, considerable evidence has accumulated arguing that strain-specified properties are enciphered in the conformation of PrP^{Sc} (10, 37, 42, 50, 65, 74). The lack of an atomic structure for PrP^{Sc} (26) has prevented comparison of the detailed structures of PrP^{Sc} molecules enciphering different strains.

Studies of synthetic prions combined with investigations of naturally occurring strains support the thesis that prions are devoid of a prion-specific nucleic acid. A novel strain of prions was produced using mouse (Mo) recombinant PrP composed of residues 89 to 230 (36). The first synthetic prion strain (MoSP1) was inoculated into transgenic 9949 mice expressing N-terminally truncated MoPrP(Δ23–88) and wild-type FVB mice expressing full-length MoPrP. Incubation time measurements, neuropathologic lesion profiles, and conformational stability studies using guanidinium HCl denaturation indicate that MoSP1 prions differ from RML and many other prion strains derived from humans with Creutzfeldt-Jakob disease, sheep with scrapie, and cattle with bovine spongiform encephalopathy (37).

In earlier UV irradiation studies, a target size of either 4 bases for a single-stranded nucleic acid or 35 to 40 base pairs for a double-stranded nucleic acid was determined (8). The upper limit of number of bases from UV irradiation studies is

similar to the maximal possible size of a putative nucleic acid of 25 bases as determined RRGE analysis (Fig. 4).

We explored the possibility that a noncoding, small nucleic acid might modulate strain-specified characteristics including the disease phenotype (77). To test this hypothesis, we studied two strains with different incubation times. If the putative small nucleic acid prolongs the incubation time of the 139H long strain compared to the Sc237 short strain, then UV irradiation should shorten the 139H incubation time. Conversely, if the small nucleic acid shortens the incubation time of the Sc237 short strain compared to the 139H long strain, then UV irradiation should lengthen the Sc237 incubation time. The incubation times of Sc237 and 139H were not altered by UV irradiation, although the titers of both strains were reduced (Fig. 5). We conclude that there is no evidence for an accessory polynucleotide of either cellular or foreign origin that modulates the strain-specified phenotype.

Small RNAs. The aim of the present study was to determine whether the maximum length of a prion-specific nucleic acid could be restricted further than the 80 bases reported earlier (31). Our approach was to maximize nucleic acid degradation while retaining as much prion infectivity as possible. Minimizing the loss of prion infectivity is critical since our conclusions are based on the premise that all nucleic acid molecules with a P/I ratio smaller than 1 cannot be essential for infectivity. We assumed, as in our earlier studies, that heterogeneous nucleic acids represent a continuous distribution of all sizes.

Until recently, the smallest nucleic acids that were known to be biologically active were viroids. Data in this paper clearly exclude any nucleic acid of a size similar than that of the viroids. But the recent recognition of small RNAs such as micro-RNAs or small interfering RNAs show that RNAs of ≈25 nucleotides can profoundly alter biological processes (62). It is noteworthy that small interfering RNAs that inhibit PrP^{C} expression in cultured cells have been reported and as such inhibit PrP^{Sc} formation (18, 75). Moreover, there is ample evidence for interaction of nucleic acids with PrP in vitro (1, 17, 23, 24, 72). However, the specificity and biological relevance of the interaction of nucleic acids with PrP is unclear. While we did entertain the possibility that micro-RNAs or small interfering RNAs might encipher prion strain-specified characteristics, the results of studies with synthetic prions as well as the UV-irradiation comparison for two prion strains (Fig. 5) make such a scenario unlikely.

Concluding remarks. The studies reported here as well as results from other recent investigations on synthetic prions and prion strains make the possibility of a prion-specific nucleic acid farfetched at best. There is no evidence for a prion-specific polynucleotide, and there is no unaccounted-for function that such a molecule would explain.

The three unprecedented manifestations of prion diseases (spontaneous, genetic, and acquired) also make it difficult to envision a prion-specific nucleic acid. The sporadic form of human prion disease is present with a worldwide incidence of 1 to 2 cases per 10^4 deaths (41). The generation of prion infectivity from wild-type recombinant PrP argues that only PrP^{C} is required for sporadic Creutzfeldt-Jakob disease (36, 37). The inherited prion diseases occur in 100% of carriers if the individuals do not die of another illness before the late onset of these disorders (15, 71). The generation of prion infectivity from a mutant synthetic PrP peptide contends that only mutant PrP^{C} is required for familial prion disease to occur (29, 76).

To accommodate a prion-specific polynucleotide in the pathogenesis of either sporadic or inherited prion disease, a nucleic acid must reside everywhere. There is no evidence for such ubiquitous disease-causing or -modifying molecules. Only the infectious prion diseases can readily accommodate a prion-specific nucleic acid, but the examples of infectious prion disease such as kuru and bovine spongiform encephalopathy often begin with sporadic cases (25, 52).

In conclusion, not only do the biophysical studies reported here and elsewhere make the possibility of a prion-specific polynucleotide increasingly remote, but recent studies of synthetic prions contend that such a nucleic acid does not exist.

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REFERENCES

1. **Adler, V., B. Zeiler, V. Kryukov, R. Kascsak, R. Rubenstein, and A. Grossman.** 2003. Small, highly structured RNAs participate in the conversion of human recombinant PrP^{Sen} to PrP^{Res} *in vitro*. J. Mol. Biol. **332:**47–57.
2. **Aiken, J. M., J. L. Williamson, L. M. Borchardt, and R. F. Marsh.** 1990. Presence of mitochondrial D-loop DNA in scrapie-infected brain preparations enriched for the prion protein. J. Virol. **64:**3265–3268.
3. **Alper, T., W. A. Cramp, D. A. Haig, and M. C. Clarke.** 1967. Does the agent of scrapie replicate without nucleic acid? Nature **214:**764–766.
4. **Alper, T., D. A. Haig, and M. C. Clarke.** 1966. The exceptionally small size of the scrapie agent. Biochem. Biophys. Res. Commun. **22:**278–284.
5. **Anfinsen, C. B.** 1973. Principles that govern the folding of protein chains. Science **181:**223–230.
6. **Baskakov, I. V., G. Legname, M. A. Baldwin, S. B. Prusiner, and F. E. Cohen.** 2002. Pathway complexity of prion protein assembly into amyloid. J. Biol. Chem. **277:**21140–21148.
7. **Bastian, F. O., and J. W. Foster.** 2001. Spiroplasma sp. 16S rDNA in Creutzfeldt-Jakob disease and scrapie as shown by PCR and DNA sequence analysis. J. Neuropathol. Exp. Neurol. **60:**613–620.
8. **Bellinger-Kawahara, C., J. E. Cleaver, T. O. Diener, and S. B. Prusiner.** 1987. Purified scrapie prions resist inactivation by UV irradiation. J. Virol. **61:**159–166.
9. **Bellinger-Kawahara, C., T. O. Diener, M. P. McKinley, D. F. Groth, D. R. Smith, and S. B. Prusiner.** 1987. Purified scrapie prions resist inactivation by procedures that hydrolyze, modify, or shear nucleic acids. Virology **160:**271–274.
10. **Bessen, R. A., and R. F. Marsh.** 1994. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J. Virol. **68:**7859–7868.
11. **Bruce, M. E., and A. G. Dickinson.** 1987. Biological evidence that the scrapie agent has an independent genome. J. Gen. Virol. **68:**79–89.
12. **Butler, D. A., M. R. D. Scott, J. M. Bockman, D. R. Borchelt, A. Taraboulos, K. K. Hsiao, D. T. Kingsbury, and S. B. Prusiner.** 1988. Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. J. Virol. **62:**1558–1564.
13. **Cairns, H. J. F.** 1950. Intracerebral inoculation of mice: fate of the inoculum. Nature **166:**910.
14. **Chandler, R. L.** 1961. Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet **i:**1378–1379.
15. **Chapman, J., J. Ben-Israel, Y. Goldhammer, and A. D. Korczyn.** 1994. The risk of developing Creutzfeldt-Jakob disease in subjects with the *PRNP* gene codon 200 point mutation. Neurology **44:**1683–1686.
16. **Chesebro, B.** 2004. Biomedicine. A fresh look at BSE. Science **305:**1918–1921.
17. **Cordeiro, Y., F. Machado, L. Juliano, M. A. Juliano, R. R. Brentani, D. Foguel, and J. L. Silva.** 2001. DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation. J. Biol. Chem. **276:**49400–49409.
18. **Daude, N., M. Marella, and J. Chabry.** 2003. Specific inhibition of pathological prion protein accumulation by small interfering RNAs. J. Cell Sci. **116:**2775–2779.

19. **Deleault, N. R., R. W. Lucassen, and S. Supattapone.** 2003. RNA molecules stimulate prion protein conversion. Nature **425:**717–720.
20. **Dickinson, A. G.** 1976. Scrapie in sheep and goats, p. 209–241. *In* R. H. Kimberlin (ed.), Slow virus diseases of animals and man. North-Holland Publishing, Amsterdam, The Netherlands.
21. **Diener, T. O., M. P. McKinley, and S. B. Prusiner.** 1982. Viroids and prions. Proc. Natl. Acad. Sci. USA **79:**5220–5224.
22. **Gabizon, R., M. P. McKinley, D. F. Groth, L. Kenaga, and S. B. Prusiner.** 1988. Properties of scrapie prion protein liposomes. J. Biol. Chem. **263:**4950–4955.
23. **Gabus, C., S. Auxilien, C. Pechoux, D. Dormont, W. Swietnicki, M. Morillas, W. Surewicz, P. Nandi, and J. L. Darlix.** 2001. The prion protein has DNA strand transfer properties similar to retroviral nucleocapsid protein. J. Mol. Biol. **307:**1011–1021.
24. **Gabus, C., E. Derrington, P. Leblanc, J. Chnaiderman, D. Dormont, W. Swietnicki, M. Morillas, W. K. Surewicz, D. Marc, P. Nandi, and J. L. Darlix.** 2001. The prion protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCP7 of HIV-1. J. Biol. Chem. **276:**19301–19309.
25. **Gajdusek, D. C.** 1977. Unconventional viruses and the origin and disappearance of kuru. Science **197:**943–960.
26. **Govaerts, C., H. Wille, S. B. Prusiner, and F. E. Cohen.** 2004. Structural studies of prion proteins, p. 243–282. *In* S. B. Prusiner (ed.), Prion biology and diseases, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Hecker, R., A. Taraboulos, M. Scott, K.-M. Pan, M. Torchia, K. Jendroska, S. J. DeArmond, and S. B. Prusiner.** 1992. Replication of distinct scrapie prion isolates is region specific in brains of transgenic mice and hamsters. Genes Dev. **6:**1213–1228.
28. **Hershey, A. D., and M. Chase.** 1952. Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol. **36:**39–56.
29. **Kaneko, K., H. L. Ball, H. Wille, H. Zhang, D. Groth, M. Torchia, P. Tremblay, J. Safar, S. B. Prusiner, S. J. DeArmond, M. A. Baldwin, and F. E. Cohen.** 2000. A synthetic peptide initiates Gerstmann-Sträussler-Scheinker (GSS) disease in transgenic mice. J. Mol. Biol. **295:**997–1007.
30. **Kascsak, R. J., R. Rubenstein, P. A. Merz, M. Tonna-DeMasi, R. Fersko, R. I. Carp, H. M. Wisniewski, and H. Diringer.** 1987. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J. Virol. **61:**3688–3693.
31. **Kellings, K., N. Meyer, C. Mirenda, S. B. Prusiner, and D. Riesner.** 1992. Further analysis of nucleic acids in purified scrapie prion preparations by improved return refocussing gel electrophoresis (RRGE). J. Gen. Virol. **73:**1025–1029.
32. **Kimberlin, R., and C. Walker.** 1977. Characteristics of a short incubation model of scrapie in the golden hamster. J. Gen. Virol. **34:**295–304.
33. **Kimberlin, R. H., S. Cole, and C. A. Walker.** 1987. Pathogenesis of scrapie is faster when infection is intraspinal instead of intracerebral. Microb. Pathog. **2:**405–415.
34. **King, C. Y., and R. Diaz-Avalos.** 2004. Protein-only transmission of three yeast prion strains. Nature **428:**319–323.
35. **Latarjet, R., B. Muel, D. A. Haig, M. C. Clarke, and T. Alper.** 1970. Inactivation of the scrapie agent by near monochromatic ultraviolet light. Nature **227:**1341–1343.
36. **Legname, G., I. V. Baskakov, H.-O. B. Nguyen, D. Riesner, F. E. Cohen, S. J. DeArmond, and S. B. Prusiner.** 2004. Synthetic mammalian prions. Science **305:**673–676.
37. **Legname, G., H.-O. B. Nguyen, I. V. Baskakov, F. E. Cohen, S. J. DeArmond, and S. B. Prusiner.** 2005. Strain-specified characteristics of mouse synthetic prions. Proc. Natl. Acad. Sci. USA **102:**2168–2173.
38. **Maddelein, M. L., S. Dos Reis, S. Duvezin-Caubet, B. Coulary-Salin, and S. J. Saupe.** 2002. Amyloid aggregates of the HET-s prion protein are infectious. Proc. Natl. Acad. Sci. USA **99:**7402–7407.
39. **Manuelidis, L.** 1996. In the community of dinosaurs: the viral view, p. 375–387. *In* L. Court and B. Dodet (ed.), Transmissible subacute spongiform encephalopathies: prion diseases. Elsevier, Paris, France.
40. **Marsh, R. F., and R. H. Kimberlin.** 1975. Comparison of scrapie and transmissible mink encephalopathy in hamsters. II. Clinical signs, pathology and pathogenesis. J. Infect. Dis. **131:**104–110.
41. **Masters, C. L., J. O. Harris, D. C. Gajdusek, C. J. Gibbs, Jr., C. Bernouilli, and D. M. Asher.** 1978. Creutzfeldt-Jakob disease: patterns of worldwide occurrence and the significance of familial and sporadic clustering. Ann. Neurol. **5:**177–188.
42. **Mastrianni, J. A., R. Nixon, R. Layzer, G. C. Telling, D. Han, S. J. DeArmond, and S. B. Prusiner.** 1999. Prion protein conformation in a patient with sporadic fatal insomnia. N. Engl. J. Med. **340:**1630–1638.
43. **McCarty, M.** 1994. A retrospective look: how we identified the pneumococcal transforming substance as DNA. J. Exp. Med. **179:**385–394.
44. **McKinley, M. P., R. K. Meyer, L. Kenaga, F. Rahbar, R. Cotter, A. Serban, and S. B. Prusiner.** 1991. Scrapie prion rod formation *in vitro* requires both detergent extraction and limited proteolysis. J. Virol. **65:**1340–1351.
45. **Mehlhorn, I., D. Groth, J. Stöckel, B. Moffat, D. Reilly, D. Yansura, W. S. Willett, M. Baldwin, R. Fletterick, F. E. Cohen, R. Vandlen, D. Henner, and S. B. Prusiner.** 1996. High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. Biochemistry **35:**5528–5537.
46. **Meyer, N., V. Rosenbaum, B. Schmidt, K. Gilles, C. Mirenda, D. Groth, S. B. Prusiner, and D. Riesner.** 1991. Search for a putative scrapie genome in purified prion fractions reveals a paucity of nucleic acids. J. Gen. Virol. **72:**37–49.
47. **Narang, H.** 2002. A critical review of the nature of the spongiform encephalopathy agent: protein theory versus virus theory. Exp. Biol. Med. **227:**4–19.
48. **Oesch, B., D. F. Groth, S. B. Prusiner, and C. Weissmann.** 1988. Search for a scrapie-specific nucleic acid: a progress report, p. 209–223. *In* G. Bock and J. Marsh (ed.), Novel infectious agents and the central nervous system. Ciba Foundation Symposium 135. John Wiley and Sons, Chichester, United Kingdom.
49. **Parry, H. B.** 1983. Scrapie disease in sheep. Academic Press, New York, N.Y.
50. **Peretz, D., R. A. Williamson, G. Legname, Y. Matsunaga, J. Vergara, D. Burton, S. J. DeArmond, S. B. Prusiner, and M. R. Scott.** 2002. A change in the conformation of prions accompanies the emergence of a new prion strain. Neuron **34:**921–932.
51. **Peretz, D., R. A. Williamson, Y. Matsunaga, H. Serban, C. Pinilla, R. B. Bastidas, R. Rozenshteyn, T. L. James, R. A. Houghten, F. E. Cohen, S. B. Prusiner, and D. R. Burton.** 1997. A conformational transition at the N-terminus of the prion protein features in formation of the scrapie isoform. J. Mol. Biol. **273:**614–622.
52. **Phillips, N. A., J. Bridgeman, and M. Ferguson-Smith.** 2000. Findings and conclusions, the BSE inquiry, vol. 1. Her Majesty's Stationery Office, London, England.
53. **Prusiner, S. B.** 2004. Development of the prion concept, p. 89–141. *In* S. B. Prusiner (ed.), Prion biology and diseases, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Prusiner, S. B.** 1991. Molecular biology of prion diseases. Science **252:**1515–1522.
55. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216:**136–144.
56. **Prusiner, S. B.** 1984. Prions. Sci. Am. **251:**50–59.
57. **Prusiner, S. B.** 1998. Prions. Proc. Natl. Acad. Sci. USA **95:**13363–13383.
58. **Prusiner, S. B., D. C. Bolton, D. F. Groth, K. A. Bowman, S. P. Cochran, and M. P. McKinley.** 1982. Further purification and characterization of scrapie prions. Biochemistry **21:**6942–6950.
59. **Prusiner, S. B., S. P. Cochran, D. F. Groth, D. E. Downey, K. A. Bowman, and H. M. Martinez.** 1982. Measurement of the scrapie agent using an incubation time interval assay. Ann. Neurol. **11:**353–358.
60. **Prusiner, S. B., M. P. McKinley, K. A. Bowman, D. C. Bolton, P. E. Bendheim, D. F. Groth, and G. G. Glenner.** 1983. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell **35:**349–358.
61. **Prusiner, S. B., J. Safar, and S. J. DeArmond.** 2004. Bioassays of prions, p. 143–186. *In* S. B. Prusiner (ed.), Prion biology and diseases, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
62. **Ramaswamy, G., and F. J. Slack.** 2002. siRNA. A guide for RNA silencing. Chem. Biol. **9:**1053–1055.
63. **Rohwer, R. G.** 1991. The scrapie agent: "a virus by any other name." Curr. Top. Microbiol. Immunol. **172:**195–232.
64. **Rose, J. A.** 1974. Parvovirus reproduction, p. 1–61. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 3. Plenum Press, New York, N.Y.
65. **Safar, J., H. Wille, V. Itri, D. Groth, H. Serban, M. Torchia, F. E. Cohen, and S. B. Prusiner.** 1998. Eight prion strains have PrP^{Sc} molecules with different conformations. Nat. Med. **4:**1157–1165.
66. **Safar, J. G., M. Scott, J. Monaghan, C. Deering, S. Didorenko, J. Vergara, H. Ball, G. Legname, E. Leclerc, L. Solforosi, H. Serban, D. Groth, D. R. Burton, S. B. Prusiner, and R. A. Williamson.** 2002. Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice. Nat. Biotechnol. **20:**1147–1150.
67. **Sammons, D. W., L. D. Adams, and E. E. Nishizawa.** 1981. Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. Electrophoresis **2:**135–141.
68. **Schaffer, F. L., and C. E. Schwerdt.** 1959. Purification and properties of poliovirus. Adv. Virus Res. **6:**159–204.
69. **Schlesinger, R. W.** 1949. The mechanism of active cerebral immunity to equine encephalomyelitis virus. I. Influence of the rate of viral multiplication. J. Exp. Med. **89:**491–505.
70. **Sparrer, H. E., A. Santoso, F. C. Szoka, Jr., and J. S. Weissman.** 2000. Evidence for the prion hypothesis: induction of the yeast [PSI^+] factor by in vitro-converted Sup35 protein. Science **289:**595–599.
71. **Spudich, S., J. A. Mastrianni, M. Wrensch, R. Gabizon, Z. Meiner, I. Kahana, H. Rosenmann, E. Kahana, and S. B. Prusiner.** 1995. Complete penetrance of Creutzfeldt-Jakob disease in Libyan Jews carrying the E200K mutation in the prion protein gene. Mol. Med. **1:**607–613.
72. **Supattapone, S.** 2004. Prion protein conversion *in vitro*. J. Mol. Med. **82:**348–356.
73. **Tanaka, M., P. Chien, N. Naber, R. Cooke, and J. S. Weissman.** 2004. Conformational variations in an infectious protein determine prion strain differences. Nature **428:**323–328.

74. **Telling, G. C., P. Parchi, S. J. DeArmond, P. Cortelli, P. Montagna, R. Gabizon, J. Mastrianni, E. Lugaresi, P. Gambetti, and S. B. Prusiner.** 1996. Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. Science **274:**2079–2082.
75. **Tilly, G., J. Chapuis, D. Vilette, H. Laude, and J. L. Vilotte.** 2003. Efficient and specific down-regulation of prion protein expression by RNAi. Biochem. Biophys. Res. Commun. **305:**548–551.
76. **Tremblay, P., H. L. Ball, K. Kaneko, D. Groth, R. S. Hegde, F. E. Cohen, S. J. DeArmond, S. B. Prusiner, and J. G. Safar.** 2004. Mutant PrP^{Sc} conformers induced by a synthetic peptide and several prion strains. J. Virol. **78:**2088–2099.
77. **Weissmann, C.** 1991. A "unified theory" of prion propagation. Nature **352:** 679–683.
78. **Wells, G. A. H., and J. W. Wilesmith.** 1995. The neuropathology and epidemiology of bovine spongiform encephalopathy. Brain Pathol. **5:**91–103.